Doctoral Thesis for the Degree of Doctor of Philosophy, Faculty of Medicine

Inflammation and Cell Proliferation Following Perinatal Brain Injury

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"One ought not to go to cadavers to study life."

C.G. Jung

ABSTRACT

Inflammation plays an important role in cerebral ischemic injury in the immature brain. The aim of the thesis was to investigate (1) the role of astrocyte activation and reactive gliosis in neonatal hypoxic-ischemic (HI) brain injury, (2) the role of complement derived peptide C3a in neonatal HI brain injury, (3) the effect of neonatal HI brain injury on fear learning and behaviour and (4) the effects of lipopolysaccharide (LPS) induced systemic inflammation on cell proliferation in the developing brain. Glial fibrillary acidic protein and vimentine deficient (*GFAP*$^{-/-}$*Vim*$^{-/-}$) mice, transgenic mice over-expressing C3a under the control of a GFAP promoter (*C3a/GFAP*) and wild type mice were exposed to HI at postnatal day 9 (P9). To induce unilateral HI, the left common carotid artery was permanently ligated followed by exposure to a gas mixture of low oxygen content. Bromodeoxyuridine (BrdU) was injected intraperitoneally (i.p.) to detect cell proliferation. Memory was tested in mice exposed to HI by using a trace fear conditioning test. We found no difference in the hemisphere or infarct volume between *GFAP*$^{-/-}$*Vim*$^{-/-}$ and wild-type mice 3 and 22 days after HI. However at P31, *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice showed an increase in NeuN^{+}BrdU^{+} (surviving newly born) neurons in the ischemic cortex compared to wild-type. *C3a/GFAP* mice had reduced loss of hippocampal volume in the ipsilateral compared to the contralateral hemisphere and a higher hippocampus/hemisphere ratio compared to the WT in the ipsilateral hemisphere. *C3a/GFAP* mice showed a higher number of newly born and surviving neurons, astrocytes and microglia in the dentate gyrus in the ischemic hemisphere compared to the wild type mice. However, a reduced number of astrocytes and microglial cells were found in the *C3a/GFAP* mice in the ipsilateral hemisphere compared to wild-type mice. C3a mRNA expression increased in the ipsilateral subventricular zone, hippocampus and cortex in *C3a/GFAP* and wild-type neonatal mice between 0 to 6 hours after HI as shown with real time-PCR. Injection of C3a peptide into the ipsilateral cerebral ventricle, in wild-type mice, 1 hour after HI, improved memory function. The trace fear conditioning test with a shock-paired tone and light showed that the control mice remembered the shock-paired context and the shock-paired light and tone while HI treated mice did not. The volume of the ipsilateral hippocampus and the amygdala was reduced in wild-type mice exposed to HI. Wild type mice injected i.p. with LPS on P9 and evaluated at P40 showed that LPS reduces cell proliferation and survival of neurons and astrocytes in the developing brain.

Conclusion: Reactive gliosis and LPS-induced systemic inflammation have negative effects on neurogenesis and cell proliferation; whereas the complement derived peptide C3a improves the outcome after neonatal HI. Early targeting treatments that increase cell survival may be important after neonatal HI and C3a could be such a potential therapeutic target in the future.

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by roman numerals:

I. Attenuation of reactive gliosis does not affect infarct volume in neonatal hypoxic-ischemic brain injury in mice
Katarina Järlestedt*, Catherine I. Rousset*, Maryam Faiz, Ulrika Wilhelmsson, Anders Ståhlberg, Hana Sourkova, Marcela Pekna, Carina Mallard, Henrik Hagberg and Milos Pekny
PLoS ONE, 2010, April, 5, 4

II. Complement-derived peptide C3a is neuroprotective in neonatal hypoxic-ischemic brain injury
Katarina Järlestedt, Catherine I. Rousset, Anders Ståhlberg, Hana Sourkova, Alison L. Atkins, Scott R. Barnum, Milos Pekny, Carina Mallard, Henrik Hagberg and Marcela Pekna
(manuscript)

III. Pavlovian fear conditioning detects hypoxic-ischemic brain injury in neonatal mice
Katarina Järlestedt, Alison L. Atkins, Henrik Hagberg, Marcela Pekna, Carina Mallard
(submitted)

IV. Detrimental effects of LPS on dividing stem cells in the newborn brain
Andrew S. Naylor, Katarina Järlestedt, Justin Dean, Henrik Hagberg and Carina Mallard
(manuscript)

TABLE OF CONTENT

ABBREVIATIONS

AIF	Apoptosis inducing factor
AMPA	α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid
APC	Antigen-presenting cell
ATP	Adenosine triphosphosphate
BrdU	5-Bromo-2-deoxyuridine
C3aR	C3a receptor
C5aR	C5a receptor
CA	Cornu ammonis
CNS	Central nervous system
CP	Cerebral palsy
CS	Conditioned stimulus
DAB	3, 3'-Diaminobenzidine
DAPI	4', 6-Diamidino-2-phenylindole
DCX	Doublecortin
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
EBM	Experimental biomedicine
GFAP	Glial fibrillary acidic protein
HI	Hypoxia-ischemia
Iba-1	Ionized calcium binding adaptor molecule 1
i.c.v	Intra cerebroventricular
IF	Intermediate filament
IL	Interleukin
i.p.	Intra peritoneal
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MASP	MBL associated serine proteases
MAP-2	Microtubule associated protein 2
MBL	Mannose-binding lectin
mGluR	Metabotropic glutamate receptor
MMP	Matrix metalloproteinase
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NeuN	Neuronal nuclei
NGF	Nerve growth factor
NMDA	N-metyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
P	Postnatal day
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
PRR	Pattern recognition receptor
SD	Standard deviation
S.E.M	Standard error of the mean
SVZ	Subventricular zone

TBS	Tris buffered saline
TLR	Toll-like receptor
TNF	Tumour necrosis factor
US	Unconditional stimulus
WT	Wild-type

INTRODUCTION

Clinical background

Perinatal hypoxic-ischemic (HI) brain injury can result in a number of neurological disorders such as cerebral palsy (CP), mental retardation, learning disabilities and epilepsy (Johnston et al., 2001). The risk of developing perinatal brain injury is about 2 in 1000 live births in term infants but much higher in preterm infants, up to 50% (Himmelmann et al., 2010; Levene et al., 1985; Thornberg et al., 1995). HI can occur for different reasons before, during or after birth. Compression of the umbilical cord or problems with the maternal health or placenta can lead to HI. In particular the newborn preterm infant might also suffer from circulatory or respiratory problems after birth that lead to HI. HI in preterm infants causes predominately white matter injury while term infants suffering from HI more often develop injuries in both grey and white matter (Volpe, 2008). At present there are no effective therapies to repair the brain once it has been injured. More research is necessary to find regenerative therapeutic strategies that can assist the recovery of the developing brain injury after HI.

Mechanisms of hypoxic-ischemic perinatal brain injury

HI brain injury develops through two stages (McLean and Ferriero, 2004; Perlman, 2006; Volpe, 2008) and the process continues over time (Nakajima et al., 2000). The first stage in the development of HI injury, primary injury, affects the local area where energy depletion is acute and leads to necrosis of the cells (Northington et al., 2001; Volpe, 2008). The second stage occurs during reperfusion, when the blood flow is restored to the brain and secondary energy depletion occurs, which leads to inflammation and apoptotic cell death (Azzopardi and Edwards, 1995; Lorek et al., 1994).

Primary injury

HI causes an acute lack of oxygen and glucose which leads to cellular energy depletion. Extensive energy depletion causes cell necrosis, while less adenosine triphosphate (ATP) depletion may not kill the cells immediately but induce delayed cell death mechanisms such as apoptosis (Skulachev, 2006; Volpe, 2008). As the ATP formation decreases, the Na^+/K^+ -pump is unable to support the resting-potential over the membrane which causes depolarisation of the neurons and a release of glutamate (Volpe, 2008). Extracellular accumulation of the excitotoxic amino acid glutamate will occur (Hagberg et al., 1987). Glutamate will bind to ionotropic N-metyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainate receptors and metabotropic glutamate receptors (mGluR) (Degos et al., 2008; Johnston et al., 2002; Volpe, 2008) that will trigger excessive calcium influx and the formation of potentially damaging molecules such as reactive oxygen species (ROS), nitric oxide (NO), nitric oxide synthase (NOS), xanthine oxidase and phospholipase A (Blomgren and Hagberg, 2006; Ferriero, 2004). This process can lead to immediate necrotic cell death with an injury area determined by the severity of the initial insult (Blomgren et al., 2003; Skulachev, 2006; Volpe, 2008).

Secondary injury and secondary energy failure

When blood flow is restored following HI, a secondary stage of injury occurs. Reperfusion normalizes the oxygen and glucose levels, the intracellular pH and cell metabolism. However, mitochondrial respiration does not completely recover and 4-24 hours later a secondary energy failure occurs, causing glutamate release, formation of reactive oxygen species and nitric oxide. Inflammatory cells such as macrophages, neutrophils and microglia become activated and release pro-inflammatory mediators that can amplify inflammation. Resident microglia migrate and proliferate and macrophages and neutrophils infiltrate the injured area in the brain (Alvarez-Diaz et al.,

2007; Bona et al., 1999; Degos et al., 2008; Denker et al., 2007; Hudome et al., 1997; McRae et al., 1995).

Cell death

Cell death mechanisms are often divided into necrosis and apoptosis. Necrosis occurs by rupture of the nuclear, mitochondrial or cell membrane due to swelling of the cytoplasm and organelles. Excitotoxicity or HI can also activate apoptosis or programmed cell death through two major pathways; the caspase-independent apoptosis-inducing factor (AIF) pathway and the caspase-dependent (intrinsic and extrinsic) pathway. The caspase-independent and caspase-dependent pathway are more important than the necrotic pathway in immature brain compared to adult brain (Blomgren and Hagberg, 2006). The caspase-independent pathway starts by translocation of AIF from the mitochondria to the nucleus and inducing chromatinolysis and DNA fragmentation (Blomgren et al., 2003; Hagberg, 2004; Kroemer and Martin, 2005). The intrinsic caspase-dependent pathway is activated through mitochondrial outer membrane permeabilization that leads to cytochrome C release. Cytochrome C activates apoptosome assembly in addition to caspase-9 and caspase-3 activation. The extrinsic caspase-dependent pathway is initiated by activation of cell death receptor Fas or tumour necrosis factor-α (TNF-α) on the cell surface. The activation of these receptors leads to activation of caspase-8, which directly activates caspase-3, the final initiating molecule of programmed cell death.

Inflammation

Inflammation can be triggered by trauma, ischemia or infection and causes symptoms such as redness, swelling, pain, and heat in the affected tissue. Inflammation is normally a healthy response that helps the body to eliminate pathogens and activate tissue regeneration and recovery. However when the

inflammation process is unregulated and becomes excessive or chronic it can cause tissue damage and impair recovery.

Inflammation in the brain

The inflammatory process after HI involves production of inflammatory mediators such as cytokines, chemokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) through activation of inflammatory cells. Up-regulation of the pro-inflammatory cytokines interleukin (IL) 1ß, IL-18 and TNF-α play an important role in the development of HI injury in the immature rodent brain (Aly et al., 2006; Hagberg et al., 1996; Hedtjarn et al., 2002; Szaflarski et al., 1995). In addition, anti-inflammatory cytokines and neurotrophic factors are released. The relative balance between these pro- and anti-inflammatory mediators is likely to be very important for the development of the injury (Kriz, 2006). Systemic infection induced by lipopolysaccharide (LPS), a bacterial cell wall component, causes inflammation in the brain (Eklind et al., 2006), and clinically, intrauterine infection is a risk factor for white matter injury and developing CP in the preterm infant (Back, 2006; Wu et al., 2003).

Cells involved in inflammation

Microglial cells are resident macrophages in the brain that modulate inflammation, regulate homeostasis and nerve growth. They are found more abundantly in grey matter than in white matter in the adult brain, and depending on the degree of activation they change their phenotype (Kim and de Vellis, 2005; Lawson et al., 1990). After neonatal HI brain injury microglial cells become activated (Bona et al., 1999; Denker et al., 2007; McRae et al., 1995) and produce IL-1, IL-18, ROS and proteases (Bagenholm et al., 1997; Hagberg et al., 1996; Hedtjarn et al., 2002; Martin et al., 1994; Piani et al., 1992; Svedin et al., 2007).

Neutrophilic granulocytes are the most abundant white blood cell type and are a part of the innate immune system. These cells are found in the neonatal brain within 24 hours after brain injury in rodents (Bona et al., 1999). However, most of these cells stay in the intravascular compartment and infiltration into the brain parenchyma is limited after HI (Benjelloun et al., 1999; Bona et al., 1999). In both adult and immature brain injury models, inhibition or depletion of neutrophils have been shown to be protective (Hudome et al., 1997; Jiang et al., 1995; Matsuo et al., 1994; Nijboer et al., 2008).

The complement system

In 1895 Jules Bordet discovered "complementing" antibodies in the plasma that recognized and eliminated pathogens. The complement system contributes to the defence against pathogens (e.g bacteria, virus-infected cells and parasites) through release of anaphylatoxins, opsonisation and lysis of pathogens, stimulation of leukocyte chemotaxis and activation of leucocytes release of inflammatory molecules. The human complement system involves more than 30 cell-bound and soluble proteins. There are approximately 20 plasma proteins which are mainly produced and secreted by macrophages, monocytes and hepatocytes (Alper et al., 1969; Cole et al., 1983; Einstein et al., 1977). These soluble complement proteins are involved in the activation and regulation of complement proteins acting as enzymes, enzyme inhibitors, or enzyme cofactors. In addition, 10 cell-bound proteins regulate complement activation or function as receptors for proteolytic fragments generated during activation of a complement cascade. The complement cascade can be activated through three pathways, the classical pathway, the mannose-binding lectin pathway or the alternative pathway (Figure 1). The final step of the complement cascade is the terminal pathway where the formation of the membrane attack complex (MAC) on the surface of the target cell leads to cell lysis. The complement system is activated in the brain of immature rodents and human infants after HI (Cowell et al., 2003; Schultz et al., 2005).

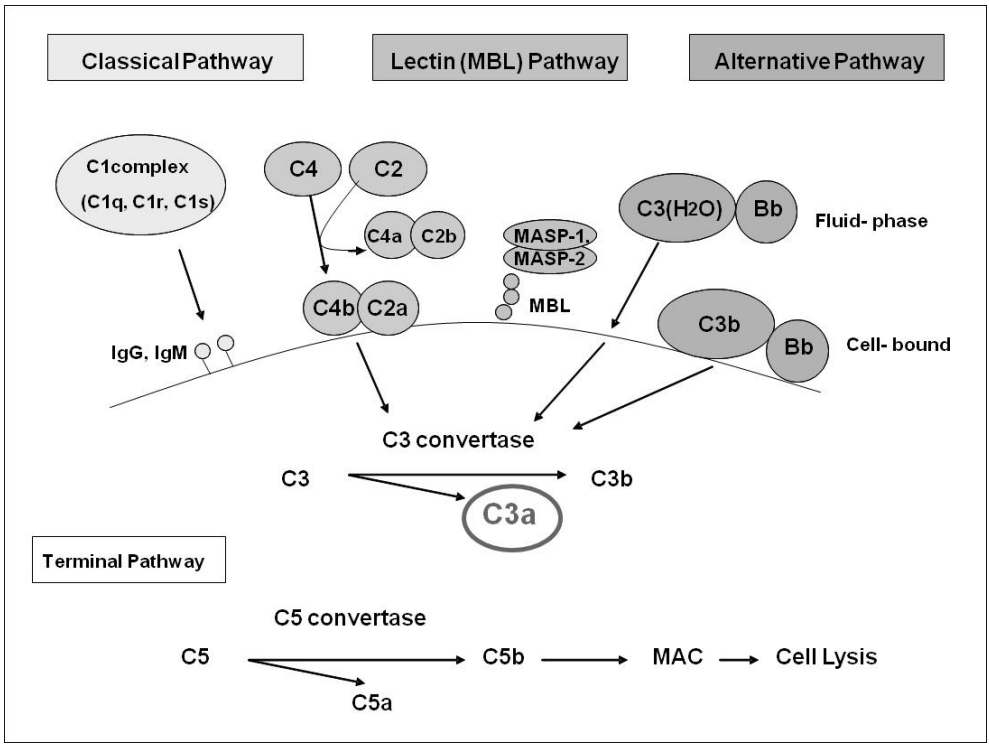

Figure 1. Schematic overview of the three pathways activating the complement system and its final step the terminal pathway that leads to cell lysis.

Activation of the complement system

The classical pathway

The complement pathway first described was the classical pathway which involves the three complement components C1 (C1q, C1r, C1s), C2 and C4. C1q is created by six identical subunits with one C-terminal globular head per subunit. At least two C1q globular heads have to bind either the Fc parts of an immunoglobulin (Ig) M molecule or two IgG molecules to activate the pathway. Upon activation the conformation of C1 changes and cleaves C4 into C4a and C4b. The thoilester bond in C4b makes it possible for the molecule to bind to a cell surface. C2 docks to the cell bound C4b and is cleaved by C1s into a large

fragment C2a and a smaller fragment C2b. The C2a complex C4b forms the C3-convertase, C4bC2a which cleaves C3 into C3a and C3b. Activation of the C1q pathway exacerbates the outcome of neonatal HI brain injury through mediating mitochondrial ROS formation and oxidative stress (Ten et al., 2010).

The Mannose-binding-lectin pathway

The mannose-binding lectin (MBL) involves the proteins MBL, C2, C4 and MBL associated serine proteases (MASP) 1 and 2. The MBL molecule shares many features with C1q (Fujita, 2002) and the classical pathway. However, the MBL pathway is an antibody independent pathway where MBL in the plasma is activated by binding to mannose on the surface of the bacteria which activate MASP 1 and 2 that cleaves plasma C4 and C2. The activation of MBL results in the formation of C3-convertase, C4bC2a that triggers the cascade. MBL can also remove apoptotic cells by opsonisation (Nauta et al., 2003).

The alternative pathway

Spontaneous hydrolysis of the thoilester bond in plasma C3 creates the C3 (H_20) molecule and activates the alternative pathway constantly. The C3 (H_20) molecule is similar to the C3b and binds to plasma factor B which cleaves plasma factor D into Ba and Bb. The Bb is attached to C3 (H_20) and forms C3 (H_20) Bb, the C3-convertase. C3b generated in plasma is quickly inactivated by the surrounding water, however if there is a pathogen near the conversion site C3b will form a membrane-bound C3-convertase with plasma factor Bb. The biological function of the Ba molecule is still unknown.

The third complement protein (C3)

The most abundant complement protein in the plasma is the third complement protein (C3), with a basal plasma concentration in humans of 1 mg/ml which increases during inflammation (Kushner et al., 1972). C3 is mainly produced and secreted by hepatocytes but is also synthesized outside the liver and is

composed of an alpha and a beta chain. The alpha and beta chains are attached to each other through a disulfide bond (Barnum et al., 1989; Fong et al., 1990; Janatova et al., 1986). The activation of the complement system causes the formation of the enzymatic complex C3-convertase. The C3-convertase cleaves the C3 alpha chain and generates C3a; a small soluble anaphylatoxic fragment that stimulates leukocyte recruitment and activation to release pro-inflammatory cytokines (Frank and Fries, 1991; Hugli, 1990). The larger fragment of the C3 molecule, C3b contains a thiolester bond which when exposed binds to target structures in the surrounding area and trigger the terminal part of the complement cascade (Sim et al., 1981). The C3 protein has been found as early as 15 weeks into gestation in the fetus (Adinolfi and Gardner, 1967), and the newborn infant C3 protein exhibits allotypic differences compared to their mothers C3, which demonstrate that the fetus produces its own C3. The C3 concentration is also very low in the infant compared to the mother which indicates that the C3 does not pass through the placenta (Propp and Alper, 1968).

C3a is neuroprotective in vitro (van Beek et al., 2001) and induces expression of nerve growth factor (NGF) in human microglial cells (Heese et al., 1998). In vivo, adult mice deficient in C3, C3a-receptor (C3aR) or treated with a C3aR antagonist show decreased neurogenesis after ischemia (Rahpeymai et al., 2006), which confirms the positive effect of C3 and C3a on neurons. Expression of C3aR1, C5aR1 and C1q mRNA is induced by HI in the immature mouse brain (Hedtjarn et al., 2004). The role of C3 and C3a in neonatal HI is unknown.

Astrocytes and reactive gliosis

Astrocytes

Glial cells are the most common cell type in the CNS and are divided into two groups: microglial and macroglial cells. Macroglial cells can be divided into

astrocytes and oligodendrocytes. Traditionally astrocytes were thought to have a star-shaped morphology. This idea was primarily based on the commonly used antibody against glial fibrillary acidic protein (GFAP) used to visualize the cells (Eng et al., 2000) which only shows about 15% of the actual cell structure. More recent studies have shown that astrocytes have a bush-like morphology (Bushong et al., 2002). Their bush-like morphology reaches up to 50 μm from the cell soma (Wilhelmsson et al., 2004). Astrocytes can communicate with each other and with other cell types in the brain such as neurons, ependymal cells, oligodendrocytes and microglia. Astrocytes are found to maintain tight control of local ion and pH homeostasis, glucose levels and provide metabolic substrates (Nedergaard et al., 2003; Ransom et al., 2003). Also, astrocytes become activated in response to inflammation and injury, they protect neurons from NO toxicity (Chen et al., 2001) and can induce and stabilize neuronal synapses (Christopherson et al., 2005; Ullian et al., 2001).

Intermediate filaments

The cytoskeleton is constructed of three parts, the microtubules, actin filaments and intermediate filaments (IFs). Microtubules provide structural support and participate in intracellular transport. Actin filaments determine cell shape and mobility. However, the function of IFs is still not entirely understood although it is known that IF networks interact with microtubules and actin. In higher vertebrates IF proteins are often cell type specific or specific for the developmental stage of the cell. The astrocyte IF composition is dependent on the developmental and activation stage of the cell. In immature astrocytes IFs are composed of vimentin, synemin and nestin (Dahlstrand et al., 1995; Pixley and de Vellis, 1984; Sultana et al., 2000), while in mature non-reactive astrocytes IFs are composed of GFAP and vimentin. During astrocyte activation GFAP and vimentin are up-regulated and nestin re-expressed (Frisen et al., 1995; Lin et al., 1995).

Reactive gliosis in brain injury

In almost all CNS pathologies in humans, such as neurotrauma, stroke, ischemia or tumours, astrocytes in the affected area will become activated. The activation process, known as reactive gliosis is triggered by cell death, inflammatory processes and plasma proteins. The reactive astrocyte morphology is altered accompanied by differential expression of hundreds of genes compared to the non-reactive astrocyte (Eddleston and Mucke, 1993; Eng et al., 2000). Activated astrocytes migrate to the injured area and contribute to glial scar formation, which mainly consists of proteoglycans and reactive astrocytes. Glial scars may act as a barrier by sealing off the injured tissue from the healthy tissue; however, axons cannot regenerate beyond glial scars, which may inhibit neuronal recovery processes after damage (Silver and Miller, 2004). Adult mice deficient in GFAP and vimentin lack intermediate filaments and develop less glial scarring (Pekny et al., 1999) which leads to increased infarction volume after ischemic injury (Li et al., 2008). However, GFAP and vimentin deficient mice develop worse injury immediately after entorhinal cortex lesion than controls but show a complete synaptic regeneration two weeks after injury (Wilhelmsson et al., 2004). These mice also show increased neurogenesis and cell proliferation in the hippocampal dentate gyrus with age (Larsson et al., 2004). The role that astrocyte activation and reactive gliosis play in neonatal HI is unknown.

Cell proliferation and recovery in the CNS

Neurogenesis after brain injury

In the mammalian brain, including humans, neurons are added throughout life to a limited extent from neurogenic stem cells located in the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus subgranular zone of the hippocampus (Alvarez-Buylla and Lim, 2004; Curtis et al., 2007; Eriksson et al., 1998). Cell proliferation is highest in the young brain and every day

many thousands of newly proliferated neurons are produced by the dentate gyrus in young adult rats (Cameron and McKay, 2001). The regenerative abilities of the adult brain are normally limited, but after stroke, ischemia or trauma neural progenitor cells start to proliferate in the neurogenic zones and migrate to the injured area (Liu et al., 1998). At the injury site the cells differentiate into neurons, astrocytes and oligodendrocytes (Arvidsson et al., 2002; Jin et al., 2003; Parent et al., 2002). The biological functionality of the newly born cells are not completely understood but newborn neurons in the hippocampus are believed to become integrated and involved in memory and spatial learning (Shors et al., 2001).

Cell proliferation after injury in the developing brain

Cell proliferation is increased in the immature brain after HI and newly born and surviving neurons are found in the damaged SVZ and striatum a few weeks after injury (Felling et al., 2006; Plane et al., 2004; Yang and Levison, 2006). Oligodendrocyte progenitor cells are very vulnerable to HI (Back et al., 2002; Ness et al., 2001), but proliferating immature and mature oligodendrocytes are found in the striatum after HI (Ong et al., 2005). Also newly born and surviving astrocytes and microglial cells are found in the hippocampus, striatum and cortex after HI in the immature brain (Ong et al., 2005; Plane et al., 2004; Qiu et al., 2007) .

AIMS OF THIS THESIS

The overall objective of this thesis was to investigate inflammatory mechanisms, recovery and cell proliferation after HI brain injury.

The specific aims of the thesis were:

1. To investigate the role of astrocyte activation and reactive gliosis in neonatal HI brain injury using GFAP and vimentin deficient mice.

2. To investigate the role of complement derived peptide C3a in neonatal HI brain injury.

3. To investigate Pavlovian fear conditioning as a method to assess brain injury after neonatal HI.

4. To investigate the effects of lipopolysaccharide (LPS) induced systemic inflammation on cell proliferation in the developing brain.

MATERIALS AND METHODS

Animals

All animals were housed at Experimental Biomedicine (EBM), Sahlgrenska Academy, University of Gothenburg, Sweden. The mice were kept in a 12-hour light-dark cycle, at a constant temperature of 21°C with free access to food and water. All animal experiments were approved by the local Animal Ethics Committee at the University of Gothenburg (ethical number 29-2006, 277-2007, 48-2009). The GFAP and vimentin deficient (*GFAP*$^{-/-}$*Vim*$^{-/-}$) mice were bred on a mixed C57BL6/129SV/129Ola genetic background as previously described (Eliasson et al., 1999; Pekny et al., 1995; Pekny et al., 1999) (I). Mice with a C57BL/6J background were genetically modified to overexpress C3a under the control of a glial fibrillary acidic protein (GFAP) promoter (C3a/GFAP) (Boos et al., 2004) and wild type littermates were used as control animals (II). Wild-type C57BL/6 mice were purchased from Charles River Laboratories (Sultzfield, Germany) (III, IV).

Hypoxic- ischemic brain injury model

On postnatal day (P) 9, HI brain injury was induced as previously described (Hedtjarn et al., 2002; Rice et al., 1981; Sheldon et al., 1998). The mice were anesthetized with isoflurane (Baxter Medical, 3 % for the induction and 1.5 % for maintenance), in a mixture of oxygen and nitrous oxide (1:1) and the anesthesia duration was less than 5 minutes. Through a small incision in the neck, the left common carotid artery was dissected and permanently ligated with prolene suture (6.0). After the surgery the incision was closed and infiltrated with lidocaine (Xylocain®, Astra Zeneca). The mice were returned to the dam for a 1 hour recovery period and were then placed in an incubator with humidified gas mixture of 10 % oxygen in nitrogen for 30 minutes at 36° C. After the hypoxic exposure the mice were returned to the dam (I, II, III).

Comment: This model of HI brain injury in neonatal rodents has been used extensively over the last 30 years (Vannucci and Vannucci, 2005). The brain injury is found in the cerebral hemisphere ipsilateral to the arterial occlusion and the degree of damage is dependent on the duration of the systemic hypoxia. In the present thesis a relatively short duration of hypoxia (30 min) has been used to induce mild injury, mainly confined to the hippocampus.

Injections

Bromodeoxyuridine (BrdU) (I, II, IV)

The mice were injected intraperitoneally (i.p.) with 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich; 25 mg/kg body weight), dissolved in 0.9 % NaCl saline twice daily from P 9 to P 12 (I, II) or twice (50 mg/kg body weight) on P 8 (24 hours before the LPS injection) or P 11 (48 hours after the LPS injection) (IV).

Comment: BrdU is a thymidine analogue commonly used to detect proliferating cells by incorporating itself into the cell DNA during the S-phase of the cell cycle (Nowakowski et al., 1989).

Lipopolysaccharide (LPS) (IV)

At P 9 or P 11, mice were injected once i.p. with lipopolysaccharide (LPS) from Escherichia coli (LPS O55:B5, Sigma-Aldrich) (1mg/kg body weight), dissolved in 0.9 % NaCl saline.

Comment: LPS is a component of the outer membrane of Gram negative bacteria that is commonly used experimentally to simulate infection. LPS induces an innate inflammatory response systemically (Lehnardt et al., 2002; Wright et al., 1990) and in the brain (Eklind et al., 2006). LPS induces inflammation via toll-like receptors (TLRs), a type of pattern recognition receptors (PRRs) that are involved in recognition of pathogens in the immune system. TLRs are expressed on the membrane of antigen-presenting cells (APCs) which can present the antigens to T cells, including on microglia.

Microglia, the main APC in the brain, expresses several types of TLRs including TLR-4 (Bsibsi et al., 2002; Nguyen et al., 2002). LPS, which is recognized by TLR-4 (Akira et al., 2001), induces a strong inflammatory response in microglia (Cai et al., 2000; Dean et al., 2010; Eklind et al., 2006).

C3a peptide (II)

The mice were given 5 µl of a 200 nM C3a peptide solution (Complement Technology Inc.) in phosphate buffered saline (PBS) or only PBS via intracerebroventricular (i.c.v.) injection with a constant flow rate of 1 µl/minute, using a CMA/100 microinjection pump (Bioanalytical Systems Inc.).

Tissue preparation

For immunohistochemical staining of paraffin sections, the animals were deeply anesthetized with thiopenthal (50 mg/ml; Pentothal® Sodium, Hospira) i.p. and intracardially perfusion fixed with 0.9 % NaCl followed by 5 % paraformaldehyde (Histofix, Histolab). The brains were quickly removed and post-fixed in 5 % paraformaldehyde and dehydrated in ethanol followed by xylene and embedded in paraffin. The brains were cut in 8 µm thick coronal sections with a microtome (Rotary Microm HM 355S, Microm International). The sections were prepared for histochemical staining by deparaffination in xylene, then in alcohol and finally boiled for 10 minutes in 0.01 M citric acid buffer (pH 6.0) (I, II, III).

For Quantitative real-time PCR, the animals were decapitated; the hippocampus, striatum and cortex from both hemispheres were dissected out and quickly frozen on dry-ice and stored at -80 °C (I, II).

For all genetically modified mice and littermates, approximately 5 mm of tail tissue was collected and frozen for genotyping (I, II).

Immunohistochemistry

Thionin/ acid fuchsin staining (II)

For hippocampus volume evaluation the sections were stained with thionin/ acid fuchsin. The sections were dipped, for 4 minutes, in thionin/toluidin solution (1 %), then washed in dH_2O and dipped in acid fuchsin (1 g/1l) solution, for 30 seconds. The sections were then washed again 6 times in dH_2O, dehydrated in 95 % ethanol, 100 % ethanol, Xylene and mounted with cover slip.

Immunohistochemical staining of paraffin sections (I, II, III)

Sections were incubated in PBS with 3 % H_2O_2 for 10 minutes, in 2 M HCl for 1 hour and then 30 minutes in PBS with 0.2 % Triton X-100 and 3 % BSA. For brain infarction area evaluation, the primary monoclonal mouse anti-microtubule-associated protein-2 antibody (MAP-2; 1:2000; Sigma-Aldrich), anti-pan-neurofilament protein antibody (SMI 312; 1:2000; Covance) (I) and anti-NeuN antibody (1:200; Chemicon) (II, III) were used with the secondary horse-anti-mouse, BA-2001 antibody (1:250; Vector Laboratories). Sections were exposed to avidin-biotin enzyme complex ABC-Elite (Vector Laboratories) for 1 hour and visualized with 3, 3-diaminobenzidine (DAB; 0.5 mg/ml) and nickel sulphate (15 mg/ml).

Fluorescent staining of paraffin sections (I, II)

For fluorescent staining the following primary monoclonal antibodies were used: mouse anti-BrdU (1:100; Dako), mouse anti-NeuN biotin conjugated (1:100; Chemicon), primary polyclonal rabbit anti-S100 (1:200; Dako) and rabbit anti-Iba-1 (1:1000; WAKO Chemicals). The primary antibody solution was left on sections over night in the dark at 4˚ C. After the sections were washed in PBS, the secondary antibody solution was added for 1 hour at room temperature. The stainings were visualized using Alexa Fluor conjugated

secondary antibodies (1:500; Invitrogen) for matching species or Streptavidin Alexa Fluor 488 antibody (1:500; Invitrogen) for biotinylated primary antibodies. Then the sections were washed in PBS and mounted with Dako fluorescent mounting medium.

Immunohistochemical staining of free floating sections (IV)

For BrdU staining, free floating, 25 µm thick sections (IV), were incubated for 30 minutes in 2 N hydrochloric acid at 37 °C, followed by 10 minutes in 0.1 M borate buffer (pH 8.5) and washed in dH_2O, for DNA dentaturation. For Doublecortin (DCX) and anti-phospho-Histone H3 staining the sections were pretreated with sodium citrate (pH 9) for antigen retrieval. Sections were incubated for 30 minutes in 0.6 % H_2O_2, blocked with 3 % normal donkey serum in 0.1 % Triton X-100, then incubated with monoclonal anti-BrdU (1:500; Nordic Biosite), polyclonal goat-anti DCX (1:250; Santa Cruz) or polyclonal phospho-Histone H3 (1:1000; Upstate Bio) overnight at 4 ºC. After washing the sections in tris buffered saline (TBS) they were placed in secondary antibody (biotinylated donkey anti-mouse antibody, biotinylated donkey anti-goat or biotinylated donkey anti-rabbit; 1:1000; Jackson ImmunoResearch Laboratories) amplified with avidin-biotin complex (Vectastain ABC Elite, Vector laboratories) and then visualized using a detection solution (DAB; 0.25 mg/ml, Saveen Biotech AB).

Fluorescent staining of free floating sections (IV)

Free floating sections were incubated for 72 hours at 4 ºC in primary antibodies, against BrdU (1:250, Nordic Biosite), NeuN (1:500, Chemicon), S100ß (1:500, Swant) and Iba-1 (1:500 WAKO Chemicals) from different animal species. Sections were then washed and the staining visualized using Alexa Fluor conjugated secondary antibodies (1:1000; Molecular Probes, Eugene, OR). Sections were mounted on slides in fluorescent medium

containing 4', 6-diamidino-2-phenylindole (DAPI) (DAPI Pro-Long Gold anti-fade reagent, Molecular Probes) that stains the cell nuclei.

Histomorphological evaluation

Images of MAP-2 (I), thionin/ acid fuchsin (II) or NeuN DAB (II, III) stained brain sections were obtained using a CCD camera (Olympus DP50) connected to a microscope (Optiphot-2, Nikon). Olympus Micro Image, v4.0 software was used to delineate brain regions. For paper I, MAP-2 positive and negative areas of contralateral and ipsilateral hemisphere were measured throughout the brain at a section interval of 320 µm. For Paper II, the ipsi- and contralateral hemisphere and hippocampus were measured in thionin/ acid fuchsin stained sections throughout the brain at a section interval of 320 µm. For paper III, the ipsi- and contralateral hippocampus and amygdala were measured in NeuN stained sections throughout the structures at an 80 µm interval. The infarct volume was assessed as the MAP-2 negative area in the ipsilateral hemisphere and the ipsilateral hemisphere volume was assessed as the MAP-2 negative area plus the ipsilateral MAP-2 positive area (I). All volumes were calculated according to the Cavalieri's principle where $V=\sum A \times P \times T$ (Svedin et al., 2007). Surrounding the infarct area on S100 stained sections, the length of the longest cellular processes of cortical astrocytes was measured using a Leica DM 6000B microscope with a 40x objective and Stereoinvestigator 9 (MicroBrightField System, Inc) as previously described (Wilhelmsson et al., 2004) (I).

Light microscope cell counting (II, IV)

Images of the hippocampal dentate gyrus (DG) in NeuN DAB stained sections with a 160 µm section interval were obtained using a light microscope (Leica DM 6000B; Leica Microsystems) and StereoInvestigator Software V.7 (MicroBrightField System, Inc). Images were then analysed by the MetaMorph® software (Molecular Devices) (II). To determine the total number of BrdU, DCX and phospho-Histone H3 stained cells in the DG a stereological

light microscope with a motorized stage (Leica DM 6000B; Leica Microsystems) was used. All cell counts were performed according to stereological principles using StereoInvestigator Software V.7 (MicroBrightField System, Inc) (IV).

Confocal scanning laser microscope cell counting (I, II, IV)

Using a confocal microscope (Leica TCS SP2, Leica Microsystems, Germany) the number of fluorescent double stained NeuN/BrdU, Iba-1/BrdU and S100ß/BrdU cells were counted. For paper II and IV, the total number of cells was counted in the hippocampal DG. For paper I and II, images from the subventricular zone (SVZ), through the dorsal striatum and laterally to the cortex were obtained and ImageJ 1.40 (Image Processing and Analysis in Java) software was used to analyse the Z-series stacks. The total number of double stained fluorescent cells was counted on 8 µm thick stacks of confocal images within the 450 µm x 300 µm optical fields in the respective brain regions.

Comment: Stereology based on geometrical (Cavalieri's principle) and statistical principles, calculate a three-dimensional interpretation from two-dimensional parallel sections of the brain. Throughout the thesis unbiased stereological methods have been used to determine cell numbers and volume of brain tissue. Such methods substantially reduce potential experimenter error and bias associated with morphometric analysis (Gundersen et al., 1988).

Quantitative real-time PCR

Wild-type mice were decapitated at 0 hours, 6 hours, 24 hours, 3 days, 7 days and 21 days after HI. The hippocampus, striatum and cortex from both hemispheres were dissected out, quickly frozen on dry-ice and stored at -80 ºC. Total RNA was extracted using RNeasy Lipid Tissue Mini Kit, including DNase treatment (Qiagen). RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and RNA integrity was checked on random samples using the Agilent 2100 bioanalyzer (Agilent Technologies). SuperScript III (Invitrogen) was used to performed

reverse transcription according to the instructions of the manufacturer using a mixture of 2.5 µM oligo (dT) and 2.5 µM random hexamers (both Invitrogen) as primers. Temperature profile for reverse transcription was 25 ºC for 5 minutes, 50 ºC for 60 minutes, 55 ºC for 15 minutes, and 70 ºC for 15 minutes. Real-time PCR measurements were performed using a LightCycler 480 (Roche Diagnostics). Temperature profile of real-time PCR was 95 ºC for 3 minutes, followed by 50 cycles at 95 ºC for 20 seconds, 60 ºC for 20 seconds, and 72 ºC for 20 seconds. Ten-microliter reactions contained iQ SYBR Green Supermix (Bio-Rad) and 400 nM of each primer (Eurofins MWG Operon). The following primer sequences were used:

C3 fwd	GCCTCTCCTCTGACCTCTGG
C3 rev	AGTTCTTCGCACTGTTTCTGG
C3a fwd	TCAGTACAGTTGATG
C3a rev	TGGGCATGGAGTGGCAACTT
C3aR1 fwd	TGTTGGTGGCTCGCAGAT
C3aR1 rev	GCAATGTCTTGGGGTTGAAA
GFAP fwd	AACCGCATCACCATTCCT
GFAP rev	CGCATCTCCACAGTCTTTACC
IL-6 fwd	TTCCATCCAGTTGCCTTCTT
IL-6 rev	GGTAGCATCCATCATTTCTTTGT
TNF-α fwd	TCCCTCCAGAAAAGACACCA
TNF-α rev	CCACAAGCAGGAATGAGAA

Reference genes were evaluated using the Mouse Endogenous Control Gene Panel (TATAA Biocenter) and NormFinder (Andersen et al., 2004). All data were geometrically averaged against *Pgk1* and *B2m*. Correctly sized PCR products formation was confirmed by agarose gel electrophoresis (2%) for all assays and melting curve analysis for all samples. Data analysis was performed as previously described (Nolan et al., 2006; Stahlberg et al., 2005) (I, II).

Behavioural experiments

During fear conditioning tests mice were placed in an automatic reflex conditioner box (Ugo Basile, Cat. No. 7530) adapted for fear conditioning to deliver programmable light cues and/or auditory cues and electrical stimulus to the floor bars of the cage. Auditory cue was programmed at 80 dB and 670 Hz and the electrical shock at 0.5 mA. To strengthen the association between the conditioned cues and the stimuli the mice were kept in the conditioning box for 30 seconds after the shock. Freezing was defined as a complete lack of movement. Freezing behaviour was quantified by scoring presence or absence of freezing once every 10 seconds for 2 minutes and percentage of freezing was calculated (II, III).

Fear conditioning with shock-paired tone and light (II, III)

On P 49 and P 50, fear conditioning with shock-paired tone and light was performed. Freezing behaviour was scored on P 49 for 2 minutes for baseline, then the tone and light were turned on for 20 seconds, followed by a 2-second delay and then a 2-second shock. On P 50, freezing behaviour in the conditioned environment was scored for 2 minutes before the tone and light were presented for 30 seconds without shock and freezing behaviour was measured again for 2 minutes.

Trace fear conditioning with shock-paired tone (III)

The procedure for the trace fear conditioning test with shock-paired tone was the same as described above, except that the light cue was removed and the time interval between tone and shock was increased to a 20-second delay.

Fear conditioning with shock-paired tone and additional contextual cues (III)

The fear conditioning test was performed with shock-paired tone in the shock-paired context as described above however; it was performed in a novel, non-shock-paired context. The tone was paired with the shock and additional shock-

paired contextual cues were added. These additional contextual cues consisted of a black and white striped visual cue on the back wall of the cage, lemon odour on filter paper under the cage floor, and a 50-mL glass beaker in the corner of the cage. The day after conditioning, freezing behaviour was measured both in shock-paired context and in the novel context. First, freezing behaviour was measured in the presence of the shock-paired contextual cues for 2 minutes. The shock-paired tone was then presented for 30 seconds, and freezing behaviour was measured for 2 minutes. Mice were returned to the home cage for 2 hours then placed in the novel context. The novel context cues were a solid black visual cue on the back wall of the cage, chocolate odour on filter paper in the tray under the cage floor, and a plastic sheet covering the metal bars on the floor of the cage. Freezing behaviour was measured for 2 minutes then the shock-paired tone was presented for 30 seconds, and freezing was measured again for 2 minutes.

Comment: Pavlovian fear conditioning is a commonly used test to investigate learning ability in rodents. Through teaching rodents to predict aversive events we can assesses hippocampal- and amygdala-dependent learning (Maren, 2008). The animal will learn through being presented a neutral stimulus (becomes a conditioned stimulus, CS) such as a tone, a light or a contextual cue together with an aversive unconditional stimulus (US) such as a foot shock. A foot shock elicits freezing behaviour in fearful rodents which makes it possible to measure the fear reaction when presented with the CS.

Statistics

The Student t-test (were normal distribution of data) or the Mann Whitney U test was used for statistical analysis between different time points or between the animal groups (GraphPad Prism). Kruskal-Wallis one way ANOVA followed by post hoc comparisons using a Dunn's Multiple Comparison test was used for all statistical analysis between more than two groups (GraphPad Prism). The Wilcoxon signed rank test was used to compare data from the

same animal. Results were given as mean ± standard deviation (SD) or mean ± S.E.M. Statistical differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

Attenuation of reactive gliosis does not affect the hemisphere or infarct volume, but increases the number of surviving newborn neurons after HI in the developing brain (I)

To investigate the role of reactive astrocytes in response to HI brain injury in the immature brain, we subjected GFAP and vimentin deficient mice (Pekny et al., 1999; Pekny and Nilsson, 2005) to neonatal HI. Reactive astrocytes play a critical role in protecting the ischemic penumbra in the adult brain (Li et al., 2008). After HI astrocytes undergo morphological and functional changes starting 8 hours after the insult (Sen and Levison, 2006; Sullivan et al., 2010). Our data showed a near three fold up-regulation of GFAP in astrocytes starting as early as 24 hours after the HI insult which implies that astrocytes play a major part in the pathophysiological events that lead to brain injury. However, attenuation of reactive gliosis did not have any effect on the infarct volume or the volume of the injured hemisphere detected 3 days nor 22 days after HI. It also did not change the axonal morphology, assessed with neurofilament protein staining, around the injury site. The differences in response to injury between the immature and mature brain shows that the astrocyte involvement in ischemic brain injury is dependent on the developmental stage of the brain. HI stimulated the production of neurons in the wild-type mice and attenuation of reactive gliosis resulted in a higher number of newly born and surviving neurons after HI. *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice have earlier shown increased basal hippocampal neurogenesis and cell proliferation in aged animals (Larsson et al., 2004). Attenuated reactive gliosis also increased survival and integration of neuronal grafts in the CNS (Kinouchi et al., 2003; Widestrand et al., 2007). The absence of reactive gliosis seems to benefit neurogenesis and survival of newly formed neurons after HI. Interestingly, in wild-type mice undergoing reactive gliosis, a higher loss of astrocytes after HI occurs compared with

GFAP$^{-/-}$*Vim*$^{-/-}$ which suggests that astrocyte reactivity makes the cell more vulnerable to ischemic stress.

Increasing C3a attenuates brain injury in a mouse model of perinatal HI (II)

Activation of the complement system has been thought to cause tissue damage in a number of ischemia models and to increase post-ischemic cerebral infarct volume and atrophy in adult and neonatal rats (Cowell et al., 2003; Figueroa et al., 2005). However, the role of different proteins of the complement system can vary dramatically between the immature and adult brains in ischemic injury. Neonatal C1q deficient mice, lacking the initial component of the classical pathway of complement activation, are protected against HI (Ten et al., 2005) but not the adult mice (Ten et al., 2010). Complement derived peptide C3a has anti-inflammatory effects (Kildsgaard et al., 2000) and is protective against excitotoxicity-induced neuronal death (van Beek et al., 2001). Using transgenic *C3a/GFAP* mice, which over-express C3a under the control of the GFAP promoter, we found a 10-fold increase in C3a mRNA expression in the ischemic hippocampus 6 hours after neonatal HI insult in the *C3a/GFAP* mice and a 14-fold increase in C3a mRNA expression in the ischemic cortex at the same time point. The *C3a/GFAP* animals had a 50% larger hippocampal volume compared to hemisphere volume in the injured hemisphere than the wild-type animals 22 days after HI. Our results support the belief that C3a has neuroprotective functions in the injured immature brain. We found about 40% more NeuN positive neurons in the DG after HI. Further, we found that *C3a/GFAP* mice had a larger number of newly born and surviving hippocampal neurons (NeuN^{+}BrdU^{+}) after HI compared to wildtype animals, which is in agreement with previous studies showing that activation of the C3a receptor stimulates neurogenesis in adult mice (Rahpeymai et al., 2006). Also, the *C3a/GFAP* mice had 60% higher proliferation and survival of microglia and double the amount of astrocytes in the DG after HI. To further study the

beneficial effects of C3a we injected wildtype mice i.c.v. with C3a peptide 1 hour after HI, which improved the HI induced memory deficit by 67%.

Pavlovian fear conditioning is a sensitive method to detect memory impairments following HI injury in neonatal mice (III)

We wanted to investigate the effect of HI on learning and memory by performing a fear conditioning test weeks after neonatal HI insult. Pavlovian fear conditioning tests have been extensively used in different animal models of brain injury; however, not in the commonly used neonatal HI model used in this thesis. In the fear conditioning test mice subjected to HI did not remember the association between the visual and auditory CS and the shock while the control mice did. The results show that the memory deficits caused by neonatal HI brain injury can be detected in early adulthood. However, if the time between the auditory CS and the shock were longer (20 seconds) none of the mice remembered the association between the CS and the shock. In contrast, in other brain injury models, in adult and neonatal rats, both trace and brief delay fear conditioning were affected (Chowdhury et al., 2005; Raman et al., 2008). Also, dorsal hippocampus lesions attenuated freezing behaviour with a 20-second trace between tone and aversive shock in adult rats (Chowdhury et al., 2005). Differences in experimental set-up, age of animals, and injury model can affect the memory and behaviour of the mice differently.

We added more salient contextual cues to study the degree of impact the environment plays on learning after HI. The control mice showed enhanced freezing where additional cues were used both in day 1 and day 2 observations- the shock-paired context and the non-shock-paired context after CS presentation. The mice with HI injury showed impaired ability to form associations between the shock and the CS (visual plus auditory or auditory) but their ability to remember the associations between the shock and the shock-paired contextual cues was not affected. It has been shown that both fear conditioning to discrete shock-paired stimuli such as tone or light and to

contextual cues are amygdala-dependent and that the dorsal hippocampus has been associated with contextual fear conditioning (Kim and Fanselow, 1992; Maren et al., 1997; Phillips and LeDoux, 1992). Our results showed a reduction in both amygdala and hippocampal volume in the mice exposed to neonatal HI injury and the memory impairment of these animals may be the consequence of simultaneous injury to hippocampus and amygdala.

LPS induced inflammation has different effects on cell survival and proliferation in the immature hippocampus (IV)

Lipopolysaccharide is a strong inducer of inflammation and affects immune cells in the brain of rodents at different ages (Qin et al., 2007; Spencer et al., 2007; Wang et al., 2007). Inflammation decreases cell proliferation and neurogenesis in the prenatal and adult brain (Cui et al., 2009; Ekdahl et al., 2003; Monje et al., 2003). The effect of inflammation on neurogenesis might be mediated through toll-like receptors (TLR) in the adult brain, including the LPS receptor TLR-4 (Rolls et al., 2007). Our results showed that LPS had a strong negative effect on cells born after, but not on cells born during or before LPS exposure. LPS seems to affect hippocampal cell survival rather than cell proliferation. Perhaps newborn cells that have completed cell division cycle are more prone to survive inflammation. We did not find any effect on numbers of immature neurons or cell proliferation 30 days after LPS exposure which also points to LPS having an effect on cell survival rather than proliferation. Increased cell death in the dentate gyrus, but no effect on cell proliferation after LPS has been shown in the adult brain (Bastos et al., 2008) which supports our results. Astrocytes in the hippocampus actively regulate neurogenesis by promoting proliferation and differentiation of neural stem cells (Song et al., 2002). It has been previously shown that LPS exposure reduces the number of astrocytes in fetal sheep (Mallard et al., 2003). Our results showed reduced survival of both astrocytes and neurons born two days after LPS administration. We did not find any difference in number of newborn or resident microglia in the granule cell layer two days after LPS administration

and the number of cells was very low. Perhaps microglia are more prominent in other regions of the hippocampus than the granule cell layer which was seen in a study where E coli injection lead to increased number of resident microglia and proliferating microglia in the CA1 and CA3 region of the hippocampus, but not in the dentate gyrus (Bland et al., 2010). Two days after LPS exposure might also be too early to detect microglia proliferation.

CONCLUDING REMARKS

- In this model of P 9 HI injury the intermediate filaments GFAP and vimentin do not affect injury volume, but the reduction of reactive gliosis increases the number of surviving newly born neurons in the ischemic cortex. In contrast, to the adult brain, reactive gliosis does not appear to play a major role in the protection of the neonatal brain after HI injury. Further investigation into injury mechanisms mediated by other inflammatory cell types in the immature brain is needed.

- We have shown that neonatal HI leads to increased C3a mRNA expression, and that over expression of C3a peptide reduces brain injury; coincident with increased cell proliferation and survival in the hippocampal DG. Additionally, supplementing C3a improves memory function after neonatal HI injury. More research is needed to understand how enhancing an inherent immunomodulatory pathway, such as complement/ C3a could be used as a potential therapeutic target in the future.

- For future studies we wanted to evaluate an effective behavioural test to assess neurological outcome and memory function after brain injury. Neonatal HI reduces the volume of the hippocampus and the amygdala and Pavlovian fear conditioning is a sensitive and effective method to assess learning impairment in this injury model.

- Finally, we demonstrated that LPS exposure on P 9 reduces survival of neurons and astrocytes in the developing brain. Since infection is associated with poorer neurological outcome, these results suggest early targeting treatments that increase cell survival may be important in developing effective neuroprotective strategies to improve infant outcome.

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